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Note

Association of bull semen protein estimates and SDS-PAGE profiles on semen freezability

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It is vital to identify the ejaculate with good freezability by determining the biochemical makeup of the ejaculate at the pre-freeze stage. The present study targeted to assess the use of the protein estimates and profiles at the pre-freeze stage as markers of freezability in Frieswal populations. Storing the proteins for proteomic studies is always tricky in the case of animal studies, where accessibility to liquid nitrogen is limited. Hence alternative storing approaches need to be optimized. The second part of this study examined the protein concentration and protein profiles of RNALater and frozen stored sperm cells to assess the use of RNALater preservation in sperm proteomic studies. Sperm and seminal plasma protein concentrations were quantified using Bradford assay, and total protein quantities were derived. The seminal plasma and sperm protein profiles were generated with SDS-PAGE. The protein estimates and SDS-PAGE profiles of good and poor freeze-groups were similar. Also, sperm and seminal plasma protein concentration were not correlated with the semen volume and sperm count. Even though the yield was comparatively less, the protein profiles of sperm preserved by RNALater were similar to that of frozen sperms. The present study results indicate that the protein estimates and qualitative profiles of sperm and seminal plasma proteins may not be sufficient to reveal the differences in the proteome of good and poor freezable bulls at the macro level. Hence, the protein estimates and profiles of neat semen may not be helpful for the prediction of freezability at the pre-freeze stage. Secondly, this study indicates that RNALater preservation helps store sperms for proteome analysis studies.

Keywords: Bradford Assay, Cryopreservation, Frieswal bulls, Sperm and seminal plasma proteins, SDS-PAGE, Semen

Introduction

Semen is the combination of seminal fluids and sperms. Seminal plasma is a complex fluid containing organic and inorganic components[1]. They are a rich source of protein originating from accessory sex glands, and some are serum-derived from intercellular fluids[1]. They play vital roles in sperm protection and nutrient supply[2]. They are essential for the fertility of domestic animals[3]. Multiple attempts have been made to use seminal plasma proteins as functionality markers for motility, viability, freezabiltiy, longevity maintenance, sperm capacitation, and fertilization[4-8]. However, seminal plasma has both inhibitory and stimulatory effects over sperm[2].

It is vital to identify the ejaculate with good freezability by determining the biochemical makeup of the ejaculate at the pre-freeze stage[2].Sperm freezability is highly varied between the ejaculates as influenced by multiple factors[9]. During cryopreservation, the freeze/thaw process causes inevitable structural and functional alterations in sperms[10]. Even then, some bull semen is tolerant to this stress[9]. This shows the possibility of discovering a specific marker for sperm freezability, sperm motility, membrane integrity, *etc*., boosting the livestock industry[11].

Since the sperm proteome is remodeled after ejaculation under the influence of seminal plasma[12]it is crucial to study sperm and seminal plasma protein profiles in cryopreservation. Recent comprehensive proteomic studies of sperm and seminal plasma have demonstrated the use of large-scale proteomics in discovering potential protein markers for predicting semen freezability in cattle[9,13]. However, considering the between-ejaculate variations of semen composition[13] and lack of consensus on the reported significant sperm[11,14] and seminal plasma[13]proteins in cryo freezability, one may speculate a population-specific protein involvement in cryopreservation. SDS-PAGE is a simple approach and possibly the most widely used, economical, and easy-to-perform proteomic technique today[16]. Even though simple approach, it effectively separates multiple proteins in a single sample from a complex mixture[17].Hence, the present study targeted to assess the use of the protein estimates and profiles at the pre-freeze stage as markers of freezability in Frieswal populations.

Use of RNAlater—a non-toxic solution primarily used to stabilize the RNA content of samples—in tissue preservation for proteome analysis recently described as

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equally reliable with preservation of leaf tissues[18]. RNAlater contains high concentrations of quaternary ammonium sulfates and cesiumsulfates, which denature RNases, DNases, and proteases. This solution is originally developed for RNA preservation, and its comprehensive evaluation as a protein preservative is scanty[16]. Hence, the second part of this study examined the protein concentration and protein profiles of RNALater and frozen stored sperm cells to assess the use of RNALater preservation in sperm proteomic studies, which is not available in the literature to the best of our knowledge.

Materials and Methods

Animals and grouping of bulls

The animals, semen samples, and grouping of bulls are the same as detailed in our previous report[20]. In brief, 22Frieswal (5/8 HF × 3/8 Sahiwal) bulls of 3-6 years of age were initially evaluated for their seminal parameters: initial motility, concentration, volume, and post-thaw motility (PTM) using standard semen procedures of the laboratory. A total of 450 ejaculates with an average of 22 ejaculates, ranging from 10 and 33 per bull, were recorded from these animals. All the bulls are maintained under identical management at the Central Institute for Research on Cattle, India, were used in this study. All the procedures were followed after the ethical approval of IAEC as per CPCSEA guidelines. A total of fourteen bulls with highest and lowest PTM%, (42.544 ± 1.14 (Good) *vs*.34.248 ± 1.33 (Poor)) but showed similar initial motility (72.666± 0.6 (Good) *vs*. 72.031± 0.7 (Poor)) were selected and grouped as Good (n=6) and Poor (n=8) freeze-groups for further analysis[20].

Sample preparation and protein estimation

Consecutive ejaculates (3 ejaculates in four days) from all the bulls of each freeze-group were collected. Ejaculates are fractionated into seminal plasma and sperm by centrifugation at 350 g for 30 min at room temperature. The sperm fractions were washed twice in phosphate-buffered saline (PBS), and the sperm pellet (50×10^6) was stored either at −80° or resuspended in RNALater (Invitrogen, Thermofisher Scientific) until further analysis. In frozen stored sperms at −80°, sperm cells were lysed in SDS lysis buffer [Tris (pH 8.0) 10 mM, NaCl 0.4 M, and EDTA (pH 8.0) 2 mM containing 0.5% SDS] for 1 h at ambient temperature along with the protease inhibitor Phenyl Methane Sulphonyl Fluoride (PMSF, 1 mM) as described with minor modifications[16]. In the case of RNALater preservation, sperms were initially recovered from RNALater as per the manufacturer's protocols and followed the same SDS lysis protocol described for frozen sperms. The sperm lysates were clarified at 10, 000 × g for 10 min. The supernatant was precipitated using the acetone method[20]and finally dissolved in nuclease-free water. Seminal plasma and sperm proteins were estimated using G-Quant Bradford Reagent (GCC Biotech, India) following the manufacturer's instructions, where the standard curves were plotted using bovine serum albumin.Total seminal plasma and sperm proteins are calculated by multiplying semen volume and sperm count, respectively[21].

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Sperm protein extracts and seminal plasma were separated in 10% SDS-PAGE. Electrophoresis was carried out with an initial voltage of 8 V/cm for 15-20 min, followed by 15 V/cm for 45 to 50 min under the Tris-glycine buffer system. The pre-stained protein marker with a size range of 11 to 245 kDa (Himedia, India) was used as a reference as described[16]. The gel was stained using 0.2% Coomassie brilliant blue G-250[16] for 30 min, followed by de-staining in methanol and glacial acetic acid several times. SDS gel photographs were analyzed using GelAnalyzer software (version: 19.1) for the determination of unknown molecular weight (MW) protein bands of bull semen and seminal plasma against the standard pre-stained protein molecular weight marker (Hi-media).Protein profiles were generated by counting the numbers of SDS-PAGE bands in duplicate gels for good and poor sperm proteins and seminal plasma. The same procedure was followed for generating protein profiles of RNALater and Frozen preserved sperm samples.

Statistical analysis

The freeze-group effects and effect of consecutive ejaculates on sperm and seminal plasma protein estimates were assessed using the PROC GLM model of Statistical Analysis Software (SAS Institute Inc., Cary, NC, USA, 2002).The correlation of both seminal plasma and sperm proteins with the semen volume (mL) and sperm count (10^6/mL) were assessed by Pearson correlation. The effect of preservation on sperm SDS-PAGE protein profiles and freeze-groups on protein profiles of seminal plasma and sperm proteins were estimated by independent sample *t*-test (two-tailed). Data were presented as least squared means (± SEM), and values were considered statistically significant when the *P*-value was <0.05.

Results

Protein estimates in spermatozoa and seminal plasma

The protein bands detected by SDS-PAGE for seminal plasma and sperms are presented (Table 1). A total of 23 sperm and 34 seminal plasma proteins were detected with the help of GelAnalyzer software. SDS-PAGE of sperm protein showed the presence of high – (178-235 kDa), medium (53-111 kDa), and low (30-49 kDa), and in the case of seminal plasma proteins showed the presence of high (215-235 kDa), medium (58-173 kDa) and low (17-51 kDa) for both good and poor category bulls. The protein estimates of seminal plasma and sperms are shown (Table 2). The protein estimates of the freeze-group did not vary significantly for any of the studied protein estimates. Similarly, no significant variation in protein estimates of consecutive ejaculates could be detected. Sperm protein concentration estimated from frozen sperms was significantly higher than the RNAlater stored sperm proteins (P <0.05) (Fig. 1A). The correlation of sperm and seminal plasma protein estimates with semen volume and sperm count showed no significant correlation. Also, sperm and seminal plasma proteins were not correlated (Fig 2).

Electrophoretic profile of sperm and seminal plasma proteins

Protein profiles of good and poor freeze groups are shown (Fig. 1B & C). SDS-PAGE resolved protein bands with a molecular size range of 17-245 kDa for sperms and seminal plasma, respectively. Both good and poor freeze-group generated similar profiles as estimated via an average number of visible bands using SDS-PAGE (Table 3). Similarly, the effect of sperm preservation (RNALater and Frozen) on protein profiles was in significant (Table 3).

Table 1 — Protein bands detected by the GelAnalyzer software (version: 19.1)

Source	Protein size bands detected
Seminal plasma proteins	<17, 20, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 40, 48, 50, 51, 58, 60, 63, 66, 68, 69, 75, 94, 100, 111, 113, 135, 136, 173, 180, 215, 245
Sperm proteins	30, 31, 32, 33, 34, 35, 36, 37, 38, 40, 43, 44, 48, 49, 54, 60, 61, 74, 75, 111, 113, 178, 235

Discussion

This study's average sperm protein concentration (0.39 mg/50 × 10^6 sperms) was comparable with other reports from cross-bred cattle[22], where authors report a protein concentration range of 5.01 to 10.47 mg/10^9 sperms. Similarly, the seminal plasma protein concentration followed the reports from Holstein bulls[23] and cross-bred bulls[22]. Comparable protein concentration was also reported using a hand-held Refractometer[24]. However, a lower protein concentration was reported when semen was collected by electroejaculation or transrectal massage[25]. These

Table 2 — Protein estimates in spermatozoa and seminal plasma

Groups	Seminal Plasma Protein Concentration (mg/mL) *	Sperm Protein Concentration (mg/mL)*	Seminal Plasma Protein (mg) *	Total Sperm Protein (mg) *
Freeze-Groups				
CS	72.29± 2.84 (11)	0.39±0.02 (6)	363.22± 30.28 (11)	7.70±1.402 (6)
NCS	73.10± 2.29 (17)	0.39±0.015 (6)	389.73± 25.108 (17)	9.56±1.23 (6)
Date-Groups				
Day 1	65.82 ± 6.25 (2)	0.321 ± .028 (2)	368.921 ±69.255 (2)	5.654± 2.542 (2)
Day 2	76.48 ± 2.45 (13)	0.392± .011 (12)	410.896 ±27.164 (13)	8.745± 1.038 (12)
Day 4	70.16 ± 2.45 (13)	0.3637± .023(5)	345.963 ±28.273 (13)	11.820± 2.24 (5)

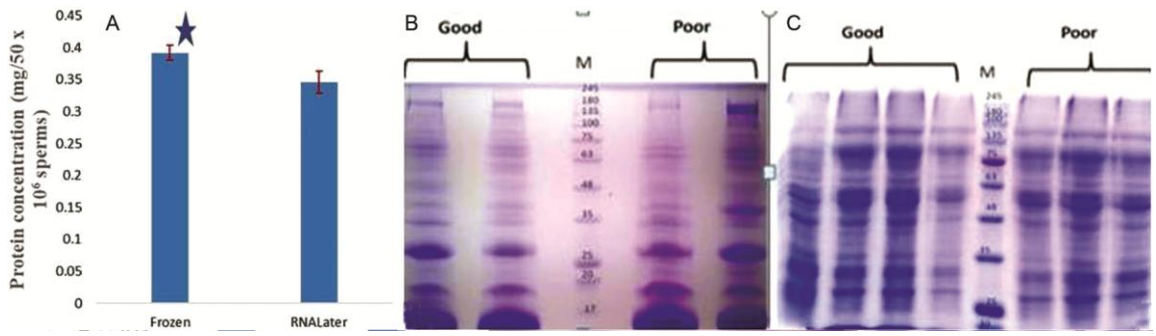

Fig. 1 — Effect of sperm preservation (Frozen *vs*.RNALater) on (A) sperm protein concentration; (B) SDS-PAGE profiles of seminal plasma; and (C) Sperm

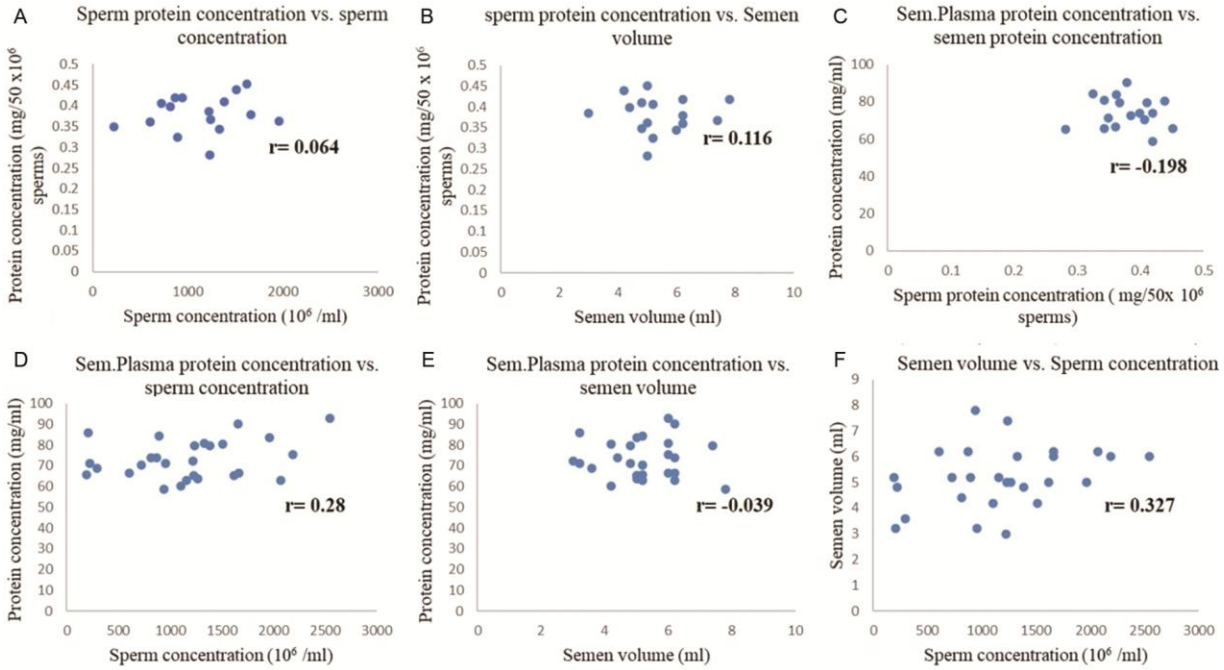

Fig. 2 — Correlation analysis (A) sperm protein concentration *vs.* sperm concentration (10^6/mL); (B) sperm protein concentration *vs.* semen volume; (C) seminal plasma protein concentration *vs.* sperm protein concentration; (D) seminal plasma protein concentration *vs.* sperm concentration; (E) seminal plasma protein concentration and semen volume; and (F), semen volume *vs.* sperm concentration

Table 3 — SDS-PAGE Protein profiles

Statistics	N	Ave. No. of Bands (Mean± SEM)
Preservation effect		
FR	5	16.40 ± 0.60
RL	5	15.40 ± 0.40
Freeze-Groups		
Sperm		
Good	5	16.00 ± 0.70
Poor	5	15.80 ± 0.37
Seminal Plasma		
Good	5	18.25 ± 1.652
Poor	5	19.00 ± 0.408

may indicate the role of the method of semen collection in protein concentration.

The present study results indicate a lack of association between semen (Seminal plasma and sperm) protein estimates on freezability. The other derived protein estimates, *viz*. total protein content of the seminal plasma and sperm, also `did not vary between freeze-groups. Similar to this study, it was reported that total protein concentration did not change after semen cryopreservation in bovine semen[23]. However, seminal plasma protein concentrations are correlated with sheep fertility[26].

The present study finds no variation in seminal plasma and sperm protein profiles between the good and poor freezable semen. The mean number of sperm protein bands reported in this study was comparable with that of HF bulls[26], while it was higher than the reported[24]. When the present study and by Almadaly *et al*.[26] directly loaded the seminal plasma to SDS-PAGE, the study of Dixit *et al.*[24] initially precipitated the sample, which may be a possible reason for the variations reported. Even though qualitative, the SDS-PAGE is a simple technique to perform and is widely used to assess the significant variations in the protein profiles and as a diagnostic assay for detecting protein changes[27]. Seminal plasma proteins separated by SDS-PAGE, showed correlation with different seminal parameters in bucks[28] with some of the semen characteristics before and after freezing in buffalo[29] and bovine fertility markers[26,30]. Authors have reported differences in the proteome of the high and low freezable groups via different approaches using 2D PAGE[31], westernblot[32] studies. Also, multiple authors have reported the changes in the proteome due to cryopreservation at small numbers of proteins[23] or a larger proteome scale[33,9]. The findings of the present study indicate the limitations of SDS-PAGE. Even though it is simple and easy to perform, its use is mostly qualitative and may not be suitable for differentiating good and poor freezable semen.

The present study did not find significant variation between consecutive ejaculates. This preliminary observation follows our previous report[20]. Using the same experimental animals and study design, we have reported that semen collection with short ejaculatory abstinence does not cause detrimental effects regarding the seminal parameters (volume, initial motility, and concentration), instead enhancing the total antioxidant capacity of the semen.

A significant reduction in the sperm protein concentration was detected when sperms were stored in RNAlater. However, no change in the protein profile could be detected. The loss of cells during the recovery of sperms from RNAlater may be a possible reason for reducing the yield but not the quality of proteins. Multiple authors, including our previous report, have indicated a negative effect of RNAlater storage on protein composition compared to fresh[20] and frozen stored cells[34], while some authors report a lack of differences[35,36]. Taken together, it is advisable to set a pilot study on the suitability of RNAlater as a preservation media for a particular tissue sample in proteomic studies.

The present study could not correlate sperm and seminal plasma protein concentration with the initial semen volume and sperm count (10^6/mL).This may indicate that the sperm and seminal plasma protein concentrations are more or less static, *i.e*., not influenced by the sperm concentration or semen volume. Similar to this study, no correlation was detected between total seminal plasma protein concentration and semen characteristics in mongrel dogs[37], between protein concentration and sperm concentration in twelve breeds of chicken[38]. However, in Barki rams, the total protein content of seminal plasma has been reported to correlate significantly with multiple semen parameters and sperm function tests[26]. The seminal plasma protein content is unrelated to sperm counts in normospermia conditions, while azoospermia conditions can alter the sperm protein content[39].

Conclusion

The protein estimates and qualitative SDS-PAGE profiles of semen from good and poor freezable bulls were similar, indicating macro-levels seminal variations are too small to utilize to predict freezability. It might necessitate an in-depth analysis of proteomes. To the best of our knowledge, this is the first study on the impact of RNAlater storage on sperm protein profile. Similar protein profiles generated between RNAlater and frozen sperm cells indicate the usability of RNAlater in sperm proteome as an alternative to frozen storage. These findings are helpful in proteome analysis of sperm samples where a storage facility (-80°) is minimal or does not exist or at a place where the proteome analysis is conducted at a remote lab than the semen collection center necessitating transport of sperm samples.

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Conflict of interest

All authors declare no conflicts of interest.

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